

## PATENT COOPERATION TREATY

PCT

NOTIFICATION OF ELECTION  
(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

Assistant Commissioner for Patents  
 United States Patent and Trademark  
 Office  
 Box PCT  
 Washington, D.C.20231  
 ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 22 February 2000 (22.02.00)	Applicant's or agent's file reference 82402-3803
International application No. PCT/CA99/00587	Priority date (day/month/year) 26 June 1998 (26.06.98)
International filing date (day/month/year) 24 June 1999 (24.06.99)	
Applicant GUY, Phillip et al	

1. The designated Office is hereby notified of its election made:

in the demand filed with the International Preliminary Examining Authority on:  
21 January 2000 (21.01.00)

in a notice effecting later election filed with the International Bureau on:  
\_\_\_\_\_

2. The election  was

was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No.: (41-22) 740.14.35	Authorized officer Marie-José Devillard Telephone No.: (41-22) 338.83.38
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## PATENT COOPERATION TREATY

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## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

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Applicant's or agent's file reference 82402-3803	FOR FURTHER ACTION		See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/CA99/00587	International filing date (day/month/year) 24/06/1999	Priority date (day/month/year) 26/06/1998	
International Patent Classification (IPC) or national classification and IPC C12N15/00			
Applicant THE UNIVERSITY OF MANITOBA et al.			

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 8 sheets.

3. This report contains indications relating to the following items:

- I  Basis of the report
- II  Priority
- III  Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV  Lack of unity of invention
- V  Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI  Certain documents cited
- VII  Certain defects in the international application
- VIII  Certain observations on the international application

Date of submission of the demand 21/01/2000	Date of completion of this report 18.05.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Hardon, E Telephone No. +49 89 2399 8414



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/CA99/00587

**I. Basis of the report**

1. With regard to the elements of the international application (Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17)).

**Description, pages:**

1.6-25	as originally filed		
2-5	as received on	16/08/2000 with letter of	08/08/2000

**Claims, No.:**

1-25	as received on	16/08/2000 with letter of	08/08/2000
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**Drawings, sheets:**

1/13-13/13	as originally filed
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2. With regard to the language, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- the language of publication of the international application (under Rule 48.3(b)).
- the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- contained in the international application in written form.
- filed together with the international application in computer readable form.
- furnished subsequently to this Authority in written form.
- furnished subsequently to this Authority in computer readable form.
- The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

**INTERNATIONAL PRELIMINARY  
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International application No. PCT/CA99/00587

the description,      pages:  
 the claims,      Nos.:  
 the drawings,      sheets:

5.  This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)): *(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)*

6. Additional observations, if necessary:

**II. Priority**

1.  This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:  
 copy of the earlier application whose priority has been claimed.  
 translation of the earlier application whose priority has been claimed.

2.  This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:  
**see separate sheet**

**IV. Lack of unity of invention**

1. In response to the invitation to restrict or pay additional fees the applicant has:

restricted the claims.  
 paid additional fees.  
 paid additional fees under protest.  
 neither restricted nor paid additional fees.

2.  This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

complied with.

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not complied with for the following reasons:  
see separate sheet

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

all parts.  
 the parts relating to claims Nos. 1-11.

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement****1. Statement**

Novelty (N)	Yes: Claims
	No: Claims 1-7, 10, 11
Inventive step (IS)	Yes: Claims 9
	No: Claims 1-8, 10, 11
Industrial applicability (IA)	Yes: Claims no objections
	No: Claims

**2. Citations and explanations**  
see separate sheet**VII. Certain defects in the international application**

The following defects in the form or contents of the international application have been noted:  
see separate sheet

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EXAMINATION REPORT - SEPARATE SHEET**

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**Section II (priority).**

1. This preliminary opinion is being established under the assumption that the entire subject-matter is entitled to the claimed priority. If this is not the case, the "P/X" document cited in the search report may have to be considered for novelty and/or inventive step in addition to the documents cited below.

**Section IV (non-unity).**

1. The present set of claims lacks unity. The following (groups of) potential inventions have been recognised:
  - i) Recombinant expression system for expressing a nonsymbiotic hemoglobin (claims 1-11).
  - ii) Method of increasing tolerance to hypoxic conditions characterised by the use of an oxygen binding protein having a low dissociation constant for oxygen (claims 12, 14, 22 and 23-25).
  - iii) Method of lowering the level of fermentation products in an organism comprising "providing" an organism having increased levels of an oxygen binding protein having a low dissociation constant for oxygen (claim 13).
  - iv) Method of increasing the oxygen uptake of an organism comprising "providing" an organism having increased levels of an oxygen binding protein having a low dissociation constant for oxygen (claim 15).
  - v) Method of improving the "agronomic properties" of a plant comprising "providing" a plant having increased levels of an oxygen binding protein having a low dissociation constant for oxygen and growing the plant (claims 16-21).
  - vi) Methods involving measuring the levels of hemoglobin expression in seed and uses thereof (claims 26, 27).

Rule 13.2 PCT stipulates that where a group of inventions is claimed the requirement of unity shall be fulfilled only where there is a technical relationship among those inventions involving one or more of the same or corresponding special technical features. "Special" technical features are those features that define a contribution which each of the inventions makes over the prior art.

The only technical feature shared by all of the presently claimed inventions is that they relate to oxygen binding proteins. Presumably no prior art document needs to

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be cited to show that oxygen binding proteins were known and that this feature alone cannot provide a common inventive concept linking these claims. Claims 1-25 furthermore share the common technical feature of being concerned with oxygen binding proteins **having a low dissociation constant for oxygen**. Duff et al., Poole et al. and Antonini et al. (documents cited in the search report) show that various oxygen binding proteins having this property were known. Similarly organisms (plants, bacteria) having **an increased level** of these proteins were known from the prior art (the transformants of Duff et al., Arredondo-Peter et al., and Jacobson-Lyon et al.). These features in themselves thus cannot define a common contribution over the prior art either. Independent claims 12, 14 on the one hand and claims 22 and 23 on the other hand could possibly be based on a common inventive concept, namely that this specific class of oxygen-binding proteins may provide an increased resistance to hypoxia. (Note that since this potential invention has not been searched a further lack of unity within the group cannot be excluded.) However, the expression system for one type of these proteins (non symbiotic hemoglobin) and the various other uses of the broader class of proteins do not seem to share a unifying inventive concept and thus have been considered separate potential inventions.

No "corresponding" special technical feature could be identified.

The applicant failed to respond in due time to the invitation to pay additional search fees. The search thus has been limited to the first (group of) inventions identified by the search examiner namely the claimed recombinant expression system for expressing a nonsymbiotic hemoglobin (claims 1-11 as originally filed). Note that the lack of novelty of the common concept in group 1 (claim 1; see also section V herein below) leads to a further lack of unity within the group of invention 1. Claims 1-11 have nevertheless all been examined.

**Section V (novelty and inventive step).**

1. Claims 1-9 have been reformulated as method claims characterised, in part, by the results to be obtained. It is, however, noted that this result will necessarily be obtained by anybody practising the method whether this is recognised by the practitioner or not. This result is thus an inherent feature or a inherent property of the method. Citing this feature therefore does not necessarily render the method

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itself novel.

2. Duff et al. (document cited in the search report) describe the expression of nonsymbiotic barley hemoglobin in *E. coli*. This document is thus detrimental to the novelty of claims 1, 2, 3, and 7, 8, 10 and 11.
3. Arrendo-Peter et al. (cited in search report) and Trevaskis et al. describe the expression of, resp. nonsymbiotic rice and *Arabidopsis* hemoglobins in *E. coli*. These documents are detrimental to the novelty of claims 1, 2 (Arrondo-Peters only), 7, 8, 10 and 11.
4. Jacobsen-Lyon et al. (document cited in the search report) describe the expression of nonsymbiotic Casuarina hemoglobin in *Lotus*. This document is detrimental to the novelty of claims 1, 2, 4, 10 and 11.
5. The remaining claims, if novel, are concerned with mere technical variations of the above matter. at present thus no inventiv step can be recognised for these claims.
6. Attention is furthermore drawn to a document by one of the inventors (R. D. Hill, Can. J. Bot. 76: 707-712, may 1998) which discusses the effects of overexpression of barley hemoglobin or suppression of the endogenous gene. The exact publication date of this document is not available to the examiner. If prepublished this document would be detrimental to the inventive activity, if not novelty, of various claims including some of the claims which were not searched (eg. claim 12; see summary and discussion of the cited document).
7. Liu et al. describe the expression of *Vitreoscilla* hemoglobin in pseudomonads. They report increased growth and product production in some strains. However, the oxygen-binding properties of the nonsymbiotic plant hemoglobins differ from the *Vitreoscilla* protein and the other hemoglobins suggested in said document. Similar positive effects on growth and product production could therefore not necessarily be expected. An inventive step is thus recognised for claim 9 in which the expression system is *P. aeruginosa*.

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8. The available prior art documents show that uncertainty existed about the exact function of nonsymbiotic plant hemoglobins. The prior art cited under points 2-4 above suggests either a role as sensors for oxygen concentration (presumably enabling the plant to adjust accordingly) or as carriers, facilitating oxygen transport. Inducibility of the corresponding genes under hypoxic conditions have been reported (see eg. discussion of Trevaskis et al.). The available information would, however, nevertheless suggest some role in survival under oxygen stress.

Although the present inventors clearly made a scientifically important contribution to the elucidation of the mechanism by which this class of proteins contributes to survival under hypoxic conditions, it is not immediately apparent how this newly gained insight could be translated into a novel and inventive patent claim.

**Section VII (sufficiency).**

1. The expression of the *Vitreoscilla* hemoglobin gene led to different results in different *Pseudomonas* strains (Liu et al., document mentioned in the search report). These results raise doubts as to whether the positive results obtained with barley hemoglobin in one *Pseudomonas* strain can reliably be obtained with other *Pseudomonas* and in particular with any other "expression system".

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that an Hb gene is present in the genome of all higher plants (Brown et al., 1984, *J Mol Evol* 21:19-32; Bogusz et al., 1988; Appleby, 1992, *Sci Progress* 76:365-398; Taylor et al., 1994; Andersson et al., 1996, *Proc Natl Acad Sci USA* 93:427-431; Hardison, 1996, *Proc Natl Acad Sci USA* 93:5675-5682).

Very little, however, is known about the function of Hb, although it has been proposed that nonsymbiotic hemoglobins may act either as oxygen carriers to facilitate oxygen diffusion, or oxygen sensors to regulate expression of anaerobic proteins during periods of low oxygen supply. The proteins from barley (Duff et al, 1997, *J Biol Chem* 272:16746-16752, incorporated herein by reference) and rice (Arredondo-Peter et al, 1997, *Plant Physiol* 115:1259-1266) and AHB1 from *Arabidopsis* (Trevaskis et al, 1997, *Proc Natl Acad Sci* 94:12230-12234) have been shown to have high oxygen avidity, with dissociation constants for oxyhemoglobin of 2.86 nM, 0.55 nM and 1.6 nM respectively, resulting in conditions whereby the free protein will remain oxygenated at oxygen concentrations far below those at which anaerobic processes are activated. Thus, while roles for Hb in the facilitated diffusion and sensing of oxygen have been proposed (Appleby, 1992), it is unlikely that these hemoglobins would function as either facilitators of oxygen diffusion or sensors of oxygen, unless the oxygen avidity was modified by interaction with another component within the cell. Thus, while Hb or Hb related proteins are found in all divisions of living organisms, their function has not been well defined.

Herein, it is shown that nonsymbiotic hemoglobins function to maintain the energy status of cells exposed to low oxygen tensions and that this property may be a common feature throughout evolution, either during exposure to hypoxia or under high energy demand.

#### SUMMARY OF THE INVENTION

According to a first aspect of the invention, there is provided a method comprising: providing a recombinant expression system capable, when transformed into an organism, of expressing a gene encoding a nonsymbiotic plant hemoglobin, which system comprises a nucleotide sequence encoding said nonsymbiotic plant hemoglobin operably linked to control sequences effective in

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said organism; transforming or transfecting said expression system into said organism; and growing said organism under conditions such that said nonsymbiotic hemoglobin is expressed, characterized in that expression of said nonsymbiotic plant hemoglobin in said organism results in elevated ATP levels in said organism compared to an untransformed control when said organism and said control are grown under conditions of limiting oxygen or high energy demand.

According to a second aspect of the invention, there are provided cells transformed with any one of the expression systems described above.

According to a third aspect of the invention, there is provided a transgenic organism whose genome has been modified to contain the expression system described above.

According to a fourth aspect of the invention, there is provided a method of increasing tolerance to hypoxic conditions comprising:

providing an organism having increased cellular levels of an oxygen-binding protein having a low dissociation constant for oxygen; and

placing the organism under hypoxic conditions,

wherein the oxygen-binding protein acts to maintain cellular energy status during the hypoxic conditions by making oxygen available for cellular metabolism at low oxygen tension.

According to a fifth aspect of the invention, there is provided a method of lowering the level of fermentation products in an organism comprising:

providing an organism having increased cellular levels of an oxygen-binding protein having a low dissociation constant for oxygen; and

reducing the level of fermentation products in the cells of the organism by maintaining cell energy status such that fermentation is bypassed.

According to a sixth aspect of the invention, there is provided a method of maintaining cellular metabolism under hypoxic conditions comprising:

providing an organism having increased cellular levels of an oxygen-binding protein having a low dissociation constant for oxygen; and

placing the organism under hypoxic conditions,

wherein the oxygen-binding protein maintains cellular metabolism status by providing oxygen for cellular metabolism.

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According to a seventh aspect of the invention, there is provided a method of increasing oxygen uptake of an organism comprising:

providing an organism having increased cellular levels of an oxygen-binding protein having a low dissociation constant for oxygen; and

exposing the organism to an oxygen-containing environment,

wherein the increased cellular levels of the oxygen-binding protein results in increased oxygen uptake.

According to an eighth aspect of the invention, there is provided a method of improving the agronomic properties of a plant comprising:

providing a plant having increased cellular levels of an oxygen-binding protein having a low dissociation constant for oxygen; and

growing the plant.

The improved agronomic properties may include germination, seedling vigour, reduced cellular levels of fermentation products, increased oxygen uptake, and increased tolerance to hypoxic conditions.

According to a ninth aspect of the invention, there is provided a method of performing skin grafts comprising:

isolating skin cells from a patient;

transfected the skin cells with an expression system comprising a nucleotide sequence encoding an oxygen binding protein having a low dissociation constant for oxygen operably linked to control sequences effective in skin cells;

culturing the skin cells such that the oxygen binding protein is expressed; and

grafting the skin cells onto a region of skin tissue attached to the patient.

According to a tenth aspect of the invention, there is provided a method of transplanting an organ from a donor to a recipient comprising:

providing an organ for transplant;

infusing the organ with an oxygen binding protein having a low dissociation constant for oxygen, thereby improving oxygen supply to the organ; and

transplanting the organ into the recipient.

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According to an eleventh aspect of the invention, there is provided a method of selecting seeds for breeding to produce seed lines having desirable characteristics comprising:

providing a representative seed of a given seed line;  
growing the seed such that the seed germinates;  
isolating an extract from the seed;  
measuring levels of hemoglobin expression within the extract; and  
selecting or rejecting the seed for further breeding based on the hemoglobin levels.

According to a twelfth aspect of the invention there is provided a method of determining if a seed has been exposed to moisture sufficient to initiate germination comprising:

providing a seed suspected of germinating;  
isolating an extract from the seed; and  
measuring levels of hemoglobin expression within the extract,  
wherein high levels of hemoglobin expression indicate that the seed is germinating.

One embodiment of the invention will now be described in conjunction with the accompanying figures in which:

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram summarizing the structures of pAS1 and pAS2 respectively.

Figure 2 is the protein immunoblot analysis of hemoglobin expression in wild-type (BMS), HB<sup>+</sup> and HB<sup>-</sup> maize cell lines with recombinant barley hemoglobin-specific antibody.

Figure 3 is a graph of the growth rate of wild-type (BMS), HB<sup>+</sup> and HB<sup>-</sup> maize cell lines under normal atmospheric conditions.

Figure 4 is a bar graph comparison of oxygen uptake by maize wild-

## CLAIMS

## 1. A method comprising:

providing a recombinant expression system capable, when transformed into an organism, of expressing a gene encoding a nonsymbiotic plant hemoglobin, which system comprises a nucleotide sequence encoding said nonsymbiotic plant hemoglobin operably linked to control sequences effective in said organism;

transforming or transfecting said expression system into said organism; and

growing said organism under conditions such that said nonsymbiotic hemoglobin is expressed,

characterized in that expression of said nonsymbiotic plant hemoglobin in said organism results in elevated ATP levels in said organism compared to an untransformed control when said organism and said control are grown under conditions of limiting oxygen or high energy demand.

2. The method according to claim 1 wherein the control sequences include a strong constitutive promoter.

3. The method according to claim 1 wherein the nonsymbiotic plant hemoglobin is barley hemoglobin.

4. The method according to claim 1 wherein the organism is a plant.

5. The method according to claim 4 wherein the plant is maize.

6. The method according to claim 5 wherein the promoter is maize ubiquitin promoter.

7. The method according to claim 1 wherein the organism is a bacteria.

8. The method according to claim 7 wherein the bacteria is an obligate aerobe.

9. The method according to claim 7 wherein the bacteria is *P. aeruginosa*.

10. Cells transformed with the expression system according to any one of claims 1 to 9.

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11. A transgenic organism whose genome has been modified to contain the expression system according to any one of claims 1 to 9.

12. A method of increasing tolerance to hypoxic conditions comprising:

providing an organism having increased cellular levels of an oxygen-binding protein having a low dissociation constant for oxygen; and

placing the organism under hypoxic conditions,

wherein the oxygen-binding protein acts to maintain cellular energy status during the hypoxic conditions by making oxygen available for cellular metabolism at low oxygen tension.

13. A method of lowering the level of fermentation products in an organism comprising:

providing an organism having increased cellular levels of an oxygen-binding protein having a low dissociation constant for oxygen; and

reducing the level of fermentation products in the cells of the organism by maintaining cell energy status such that fermentation is bypassed.

14. A method of maintaining cellular metabolism under hypoxic conditions comprising:

providing an organism having increased cellular levels of an oxygen-binding protein having a low dissociation constant for oxygen; and

placing the organism under hypoxic conditions,

wherein the oxygen-binding protein maintains cellular metabolism status by providing oxygen for cellular metabolism.

15. A method of increasing oxygen uptake of an organism comprising:

providing an organism having increased cellular levels of an oxygen-binding protein having a low dissociation constant for oxygen; and

exposing the organism to an oxygen-containing environment,

wherein the increased cellular levels of the oxygen-binding protein results in increased oxygen uptake.

16. A method of improving the agronomic properties of a plant comprising:

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providing a plant having increased cellular levels of an oxygen-binding protein having a low dissociation constant for oxygen; and  
growing the plant.

17. The method according to claim 16 wherein the improved agronomic properties include germination.

18. The method according to claim 16 wherein the improved agronomic properties include seedling vigour.

19. The method according to claim 16 wherein the improved agronomic properties include reduced cellular levels of fermentation products.

20. The method according to claim 16 wherein the improved agronomic properties include increased oxygen uptake.

21. The method according to claim 16 wherein the improved agronomic properties include increased tolerance to hypoxic conditions.

22. A method of performing skin grafts comprising:

isolating skin cells from a patient;

transfected the skin cells with an expression system comprising a nucleotide sequence encoding an oxygen binding protein having a low dissociation constant for oxygen operably linked to control sequences effective in skin cells;

culturing the skin cells such that the oxygen binding protein is expressed; and

grafting the skin cells onto a region of skin tissue attached to the patient.

23. A method of transplanting an organ from a donor to a recipient comprising:

providing an organ for transplant;

infusing the organ with an oxygen binding protein having a low dissociation constant for oxygen, thereby improving oxygen supply to the organ; and

transplanting the organ into the recipient.

24. A method of selecting seeds for breeding to produce seed lines having desirable characteristics comprising:

providing a representative seed of a given seed line;

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growing the seed such that the seed germinates;  
isolating an extract from the seed;  
measuring levels of hemoglobin expression within the extract; and  
selecting or rejecting the seed for further breeding based on the  
hemoglobin levels.

25. A method of determining if a seed has been exposed to moisture sufficient to initiate germination comprising:

providing a seed suspected of germinating;  
isolating an extract from the seed; and  
measuring levels of hemoglobin expression within the extract,  
wherein high levels of hemoglobin expression indicate that the seed is  
germinating.

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## PATENT COOPERATION TREATY

## PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference <b>82402-3803</b>	<b>FOR FURTHER ACTION</b> see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. <b>PCT/CA 99/ 00587</b>	International filing date (day/month/year) <b>24/06/1999</b>	(Earliest) Priority Date (day/month/year) <b>26/06/1998</b>
Applicant <b>THE UNIVERSITY OF MANITOBA et al.</b>		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.

It is also accompanied by a copy of each prior art document cited in this report.

**1. Basis of the report**

a. With regard to the language, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of the sequence listing :

contained in the international application in written form.

filed together with the international application in computer readable form.

furnished subsequently to this Authority in written form.

furnished subsequently to this Authority in computer readable form.

the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2.  Certain claims were found unsearchable (See Box I).

3.  Unity of invention is lacking (see Box II).

4. With regard to the title,

the text is approved as submitted by the applicant.

the text has been established by this Authority to read as follows:

**NON-SYMBIOTIC PLANT HEMOGLOBINS TO MAINTAIN CELL ENERGY STATUS**

5. With regard to the abstract,

the text is approved as submitted by the applicant.

the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the drawings to be published with the abstract is Figure No.

as suggested by the applicant.

because the applicant failed to suggest a figure.

because this figure better characterizes the invention.

1

None of the figures.

## INTERNATIONAL SEARCH REPORT

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
  
2.  Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
  
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See additional sheet

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
  
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
  
3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
  
4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1 - 11

## Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

**FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210**

**1. Claims: 1-11**

Recombinant expression system for expressing a nonsymbiotic hemoglobin.

**2. Claims: 12, 14, 22-25**

Method of increasing tolerance to hypoxic conditions characterised by the use of an oxygen binding protein having a low dissociation constant for oxygen.

**3. Claim : 13**

Method of lowering the level of fermentation products in an organism comprising "providing" an organism having increased levels of an oxygen binding protein having a low dissociation constant for oxygen.

**4. Claim : 15**

Method of increasing the oxygen uptake of an organism comprising "providing" an organism having increased levels of an oxygen binding protein having a low dissociation constant for oxygen.

**5. Claims: 16-21**

Method of improving the "agronomic properties" of a plant comprising "providing" a plant having increased levels of an oxygen binding protein having a low dissociation constant for oxygen and growing the plant.

**6. Claims: 26, 27**

Methods involving measuring the levels of hemoglobin expression in seed and uses thereof (claims 26, 27).

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 99/00587

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/00 C07K14/805

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category <sup>a</sup>	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DUFF, STEPHEN M. G. ET AL: "Expression, purification and properties of recombinant barley ( <i>Hordeum sp.</i> ) hemoglobin: Optical spectra and reactions with gaseous ligands." JOURNAL OF BIOLOGICAL CHEMISTRY, (1997) VOL. 272, NO. 27, PP. 16746-16752., XP002117100 the whole document ---	1-3,7,8, 10,11
X	ARREDONDO-PETER, RAUL (1) ET AL: "Rice hemoglobins. Gene cloning, analysis, and O2-binding kinetics of a recombinant protein synthesized in <i>Escherichia coli</i> ." PLANT PHYSIOLOGY (ROCKVILLE), (NOV., 1997) VOL. 115, NO. 3, PP. 1259-1266., XP002117101 the whole document --- -/-	1,2,7,8, 10,11

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

## \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
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\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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\*&\* document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

30 September 1999

21.01.00

## Name and mailing address of the ISA

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Fax: (+31-70) 340-3016

## Authorized officer

Hardon, E

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/CA 99/00587

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TREVASKIS ET AL: "Two hemoglobin genes in <i>Arabidopsis</i> ...." PROC. NATL. ACAD. SCI. USA, vol. 94, October 1997 (1997-10), pages 12230-12234, XP002117102 the whole document ---	1,2,7,8, 10,11
X	JACOBSEN-LYON, KARIN ET AL: "Symbiotic and Nonsymbiotic Hemoglobin Genes of <i>Casuarina glauca</i> ." PLANT CELL, (1995) VOL. 7, NO. 2, PP. 213-223., XP002117103 the whole document ---	1,2,4, 10,11
P,X	SOWA, ALEKSANDER W. ET AL: "Altering hemoglobin levels changes energy status in maize cells under hypoxia." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (AUG. 18, 1998) VOL. 95, NO. 17, PP. 10317-10321., XP002117104 the whole document ---	1-8,10, 11
A	WO 98 12913 A (BAILEY JAMES E ; BULOW LEIF (SE)) 2 April 1998 (1998-04-02) the whole document ---	1,2,4, 10,11
A	LIU, S.-C. ET AL: "Cloning and expression of the <i>Vitreoscilla</i> hemoglobin gene in pseudomonads: Effects on cell growth." APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, (1995) VOL. 44, NO. 3-4, PP. 419-424., XP002117105 the whole document ---	1,2,7-11
A	JOSHI, MEENAL ET AL: "Oxygen dependent regulation of <i>vitreoscilla</i> globin gene: Evidence for positive regulation by FNR." BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, (1994) VOL. 202, NO. 1, PP. 535-542., XP002117106 table 2 ---	1,2,7-11
A	ANTONINI, GIOVANNI ET AL: "Cyanide dissociation from the hemoglobin of <i>Parascaris equorum</i> ." BIOCHIMICA ET BIOPHYSICA ACTA, (1994) VOL. 1205, NO. 2, PP. 252-257., XP002117107 the whole document -----	1-11

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International Application No

PCT/CA 99/00587

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9812913	A 02-04-1998	US 5959187 A		28-09-1999
		AU 4502297 A		17-04-1998
		EP 0955804 A		17-11-1999



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :  C12N 15/00		A2	(11) International Publication Number: <b>WO 00/00597</b>
			(43) International Publication Date: 6 January 2000 (06.01.00)
(21) International Application Number: PCT/CA99/00587		(74) Agent: ADE & COMPANY; Patent & Trademark Agents, 1700-360 Main Street, Winnipeg, Manitoba R3C 3Z3 (CA).	
(22) International Filing Date: 24 June 1999 (24.06.99)			
(30) Priority Data: 60/090,929 26 June 1998 (26.06.98) US 60/106,638 2 November 1998 (02.11.98) US		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
(71) Applicant (for all designated States except US): THE UNIVERSITY OF MANITOBA [CA/CA]; Industry Liaison Office, 631 Drake Centre, Winnipeg, Manitoba R3T 5V4 (CA).			
(72) Inventors; and			
(75) Inventors/Applicants (for US only): GUY, Phillip [CA/CA]; University of Manitoba, 631 Drake Centre, Winnipeg, Manitoba R3T 5V4 (CA). DUFF, Stephen [CA/CA]; University of Manitoba, 631 Drake Centre, Winnipeg, Manitoba R3T 5V4 (CA). XIANZHOU, Nie [CA/CA]; University of Manitoba, 631 Drake Centre, Winnipeg, Manitoba R3T 5V4 (CA). HILL, Robert [CA/CA]; University of Manitoba, 631 Drake Centre, Winnipeg, Manitoba R3T 5V4 (CA). DURNIN, Douglas [CA/CA]; University of Manitoba, 631 Drake Centre, Winnipeg, Manitoba R3T 5V4 (CA). SOWA, Aleksander [CA/CA]; University of Manitoba, 631 Drake Centre, Winnipeg, Manitoba R3T 5V4 (CA).			

## (54) Title: NONSYMBIOTIC PLANT HEMOGLOBINS TO MAINTAIN CELL ENERGY STATUS

## A. pAS1 (Sense)



## B. pAS2 (Anti-sense)



## (57) Abstract

Nonsymbiotic hemoglobins are broadly present across evolution; however, the function of these proteins is unknown. Cultured maize cells have been transformed to constitutively express a barley hemoglobin gene in either the sense (HB<sup>+</sup>) or antisense (HB<sup>-</sup>) orientation. Hemoglobin protein in the transformed cell lines was correspondingly higher or lower than in wild type cells under normal atmospheric conditions. Limiting oxygen availability, by placing the cells in a nitrogen atmosphere for 12 hours, had little effect on the energy status of cells constitutively expressing hemoglobin, but had a pronounced effect on both wild type and HB<sup>-</sup> cells, where ATP levels declined by 27 % and 61 % respectively. Energy charge was relatively unaffected by the treatment in HB<sup>+</sup> and wild type cells, but was reduced from 0.91 to 0.73 in HB<sup>-</sup> cells suggesting that the latter were incapable of maintaining their energy status under the low oxygen regime. Similar results were observed with *P. aeruginosa* cells transformed with an Hb expression vector. It is suggested that nonsymbiotic hemoglobins act to maintain the energy status of cells in low oxygen environments and that they accomplish this effect by promoting glycolytic flux through NADH oxidation, resulting in increased substrate level phosphorylation. Nonsymbiotic hemoglobins are likely ancestors of an early form of hemoglobin that sequestered oxygen in low oxygen environments, providing a source of oxygen to oxidize NADH to provide ATP for cell growth and development. This in turn suggests that cells containing increased levels of Hb protein will survive longer under low oxygen tension or high energy demand.

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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C12N 15/00, C07K 14/805</b>		A3	(11) International Publication Number: <b>WO 00/00597</b>
			(43) International Publication Date: <b>6 January 2000 (06.01.00)</b>
(21) International Application Number: <b>PCT/CA99/00587</b>		(74) Agent: ADE & COMPANY; Patent & Trademark Agents, 1700-360 Main Street, Winnipeg, Manitoba R3C 3Z3 (CA).	
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(71) Applicant (for all designated States except US): THE UNIVERSITY OF MANITOBA [CA/CA]; Industry Liaison Office, 631 Drake Centre, Winnipeg, Manitoba R3T 5V4 (CA).			
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DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 99/00587

**A. CLASSIFICATION OF SUBJECT MATTER**  
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Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

\* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
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Date of the actual completion of the international search

30 September 1999

Date of mailing of the international search report

21.01.00

Name and mailing address of the ISA  
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Authorized officer

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**INTERNATIONAL SEARCH REPORT**

International Application No

PCT/CA 99/00587

**C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT**

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	JACOBSEN-LYON, KARIN ET AL: "Symbiotic and Nonsymbiotic Hemoglobin Genes of <i>Casuarina glauca</i> ." PLANT CELL, (1995) VOL. 7, NO. 2, PP. 213-223., XP002117103 the whole document ---	1,2,4, 10,11
P,X	SOWA, ALEKSANDER W. ET AL: "Altering hemoglobin levels changes energy status in maize cells under hypoxia." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (AUG. 18, 1998) VOL. 95, NO. 17, PP. 10317-10321., XP002117104 the whole document ---	1-8,10, 11
A	WO 98 12913 A (BAILEY JAMES E ; BULOW LEIF (SE)) 2 April 1998 (1998-04-02) the whole document ---	1,2,4, 10,11
A	LIU, S.-C. ET AL: "Cloning and expression of the <i>Vitreoscilla</i> hemoglobin gene in pseudomonads: Effects on cell growth." APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, (1995) VOL. 44, NO. 3-4, PP. 419-424., XP002117105 the whole document ---	1,2,7-11
A	JOSHI, MEENAL ET AL: "Oxygen dependent regulation of <i>vitreoscilla</i> globin gene: Evidence for positive regulation by FNR." BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, (1994) VOL. 202, NO. 1, PP. 535-542., XP002117106 table 2 ---	1,2,7-11
A	ANTONINI, GIOVANNI ET AL: "Cyanide dissociation from the hemoglobin of <i>Parascaris equorum</i> ." BIOCHIMICA ET BIOPHYSICA ACTA, (1994) VOL. 1205, NO. 2, PP. 252-257., XP002117107 the whole document -----	1-11

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA 99/00587

### Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
2.  Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See additional sheet

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1 - 11

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/CA 99/00587

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

### 1. Claims: 1-11

Recombinant expression system for expressing a nonsymbiotic hemoglobin.

### 2. Claims: 12, 14, 22-25

Method of increasing tolerance to hypoxic conditions characterised by the use of an oxygen binding protein having a low dissociation constant for oxygen.

### 3. Claim : 13

Method of lowering the level of fermentation products in an organism comprising "providing" an organism having increased levels of an oxygen binding protein having a low dissociation constant for oxygen.

### 4. Claim : 15

Method of increasing the oxygen uptake of an organism comprising "providing" an organism having increased levels of an oxygen binding protein having a low dissociation constant for oxygen.

### 5. Claims: 16-21

Method of improving the "agronomic properties" of a plant comprising "providing" a plant having increased levels of an oxygen binding protein having a low dissociation constant for oxygen and growing the plant.

### 6. Claims: 26, 27

Methods involving measuring the levels of hemoglobin expression in seed and uses thereof (claims 26, 27).

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/CA 99/00587

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9812913 A	02-04-1998	US 5959187 A AU 4502297 A EP 0955804 A	28-09-1999 17-04-1998 17-11-1999
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